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Perspective

Structure-Based Reassessment of the Caveolin Signaling Model: Do Caveolae Regulate Signaling through Caveolin-Protein Interactions?

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Caveolin proteins drive formation of caveolae, specialized cell-surface microdomains that influence cell signaling. Signaling proteins are proposed to use conserved caveolin-binding motifs (CBMs) to associate with caveolae via the caveolin scaffolding domain (CSD). However, structural and bioinformatic analyses argue against such direct physical interactions: in the majority of signaling proteins, the CBM is buried and inaccessible. Putative CBMs do not form a common structure for caveolin recognition, are not enriched among caveolin-binding proteins, and are even more common in yeast, which lack caveolae. We propose that CBM/CSD-dependent interactions are unlikely to mediate caveolar signaling, and the basis for signaling effects should therefore be reassessed.

Introduction

The caveolin signaling hypothesis is an enduring model for understanding spatial organization of signaling at the plasma membrane (Couet et al., 1997; Lisanti et al., 1995; Okamoto et al., 1998). The central tenet of the model is that signaling proteins can form direct protein-protein interactions with the scaffolding domain of caveolin (CSD) via a signature peptide sequence, termed the caveolin binding motif (CBM) (Couet et al., 1997; Oka et al., 1997) (Figure 1). The characteristic CBMs were originally identified by the screening of a phage display peptide library (Couet et al., 1997) and were subsequently found to be present in many diverse proteins that could be immunopurified with caveolin. These consensus CBMs are hydrophobic and rich in aromatic residues (ΩxΩxxxxΩ or ΩxxxxΩxxΩ or the combined sequence ΩxΩxxxxΩxxΩ, where Ω is a Phe, Tyr, or Trp residue and x can be any amino acid) (Table 1; see also Figure S1 available online). The caveolin interaction is generally suggested to have an inhibitory role on signaling. Thus, signaling proteins associated with the cytoplasmic face of caveolae were proposed to be held in an inactive state by the caveolin "brake," prior to release from caveolae upon activation (Okamoto et al., 1998).

Numerous signaling proteins have been proposed to interact with caveolin, including cytoplasmic proteins (src family kinases, trimeric G protein subunits, Ras, PPARγ, β-catenin), and single and multispan transmembrane proteins (Patched, β-adrenergic receptors [β-ARs], adiponectin receptors) (Burgermeister et al., 2011; Couet et al., 1997; Hezel et al., 2010; Ju et al., 1997; Karpen et al., 2001; Li et al., 1996; Michel et al., 1997; Mineo et al., 1997, 1998; Song et al., 1996, 1997; Toya et al., 1998; Venema et al., 1997) (Table 1; Figure S1). The hypothesis has been extended to caveolin interactions with nonsignaling proteins, including extracellular viral proteins (Benferhat et al., 2008, 2009a, 2009b; Hovanessian et al., 2004) and key autophagic regulators such as LC3 (Chen et al., 2010), and has become a paradigm for spatial regulation of signaling pathways.

Despite the elegance of the model and the wealth of literature supporting it, including indirect experimental data showing association of specific proteins with caveolin or inhibition by CSD mimetic peptides (e.g., Bucci et al., 2000), some questions have been raised (Liu et al., 2002; Pike, 2005), and a number of crucial aspects of the model have never been systematically or rigorously addressed. For example, do the putative CBMs adopt a common structure, as would be predicted by the model? Are CBMs accessible for interaction with caveolin and positioned in such a way with respect to the caveolin-containing membrane that an interaction is feasible? How common are such motifs, and are they enriched in caveolae-associated proteins? Surprisingly, a plausible molecular mechanism for the interaction of CBMs with caveolin is yet to emerge. The wealth of genomic sequence and tertiary structural information available on putative caveolin-interacting proteins now means that these questions can be definitively answered. As outlined below, the answers to these questions raise major doubts about some of the founding principles on which the caveolar signaling model is based, leading us to propose that a significant reassessment of the caveolin signaling hypothesis may be needed.

Structures of Putative Caveolin Binding Proteins Do Not Reveal a Plausible Caveolin Binding Mechanism

The putative CBM is a short, hydrophobic sequence of 8–11 amino acid residues (Table 1; Figure S1). Two physical requirements must be met if it is to function as a bona fide caveolin interaction motif. The first requirement is that a functional CBM either must lie in a disordered region of the interacting protein (becoming ordered upon caveolin interaction) or must form

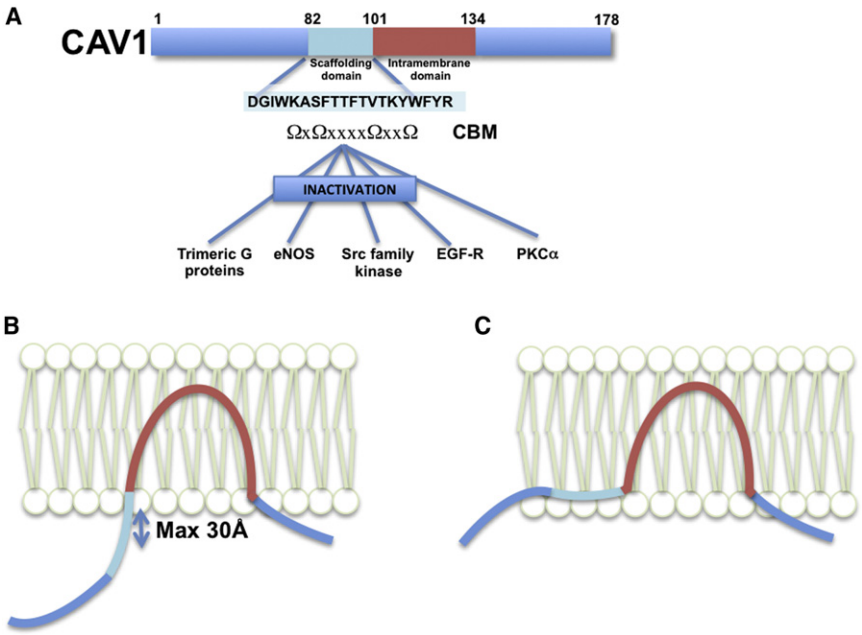

Figure 1. The Caveolin Signaling Hypothesis

(A) Schematic of the caveolin signaling hypothesis as originally proposed (Okamoto et al., 1998), with some key interacting partners highlighted. The sequence of the caveolin-1 scaffolding domain (CSD) and the consensus caveolin-binding motif (CBM) are shown.

(B and C) Two models for caveolin association with the membrane bilayer. In model (B), the CSD is exposed and shown in an extended conformation, allowing interactions with signaling proteins. However, note that the middle of the CSD is still very close to the membrane, even assuming a completely extended polypeptide conformation perpendicular to the bilayer. Model (C), in which the CSD forms part of an amphipathic cholesterol-binding in-plane helix, is an alternative model supported by a number of studies (Kirkham et al., 2008).

a common recognition structure for caveolin binding. The second requirement for a role of caveolin in sequestering proteins into caveolae is that the putative CBM should be exposed in the folded protein structure and accessible to the CSD.

We analyzed the structures of more than 40 proteins for which caveolin interactions with specific CBMs have been described (Table 1; Figure S2). Some specific examples are shown in greater detail in Figure 2 and Movie S1. This clearly reveals that no single common structural motif is adopted by the putative caveolin-interacting sequences. The CBM adopts a variety of different structures within the putative caveolin binding proteins, including extended structures, α helices, β strands, and β turns, and no consistent conformation for this peptide is observed. Even within individual protein families, including tyrosine kinases, G-protein coupled receptor (GPCRs), and protein tyrosine phosphatases, the motif adopts diverse structural orientations. For example, in the epidermal growth factor receptor (EGFR) and protein kinase C (PKC) kinase domains, the putative CBMs are found in distinct substructures, forming either a central α helix within the C-terminal lobe or a peripheral β strand on the edge of the N-terminal lobe, respectively (Figure 2). The other major observation is that these motifs are invariably found within structured regions of the proteins, often forming essential secondary structure elements. This is in distinct contrast, for example, with the recognition of multiple sorting signals and sequence motifs during formation of the analogous clathrin-coated vesicle (CCV) assembly (Owen et al., 2004; Traub, 2009). In CCVs, peptide interaction motifs are always found in structurally disordered domains and only adopt an ordered conformation upon interaction with folded domains within their partner molecule(s).

The second crucial requirement of the model, the accessibility of the CBM to interacting proteins, is also illuminated by examination of the 3D structures. As discussed above, no common structural motif is observed for the numerous putative CBM sequences. Even more tellingly, in the large majority of cases these sequences are completely inaccessible for interaction with caveolin. Figure 2 shows several different examples in which the CBM is not only inaccessible but also forms an essential part of the protein tertiary structure. Table 1 examines the solvent accessibility of putative CBMs based on the known crystal structures. The CBM sequence is hydrophobic and rich in aromatic side chains, and we find that in all cases the aromatic side chains are packed within the hydrophobic core of the putative caveolin binding proteins. By focusing on just one of these, endothelial nitric oxide synthase (eNOS), for which there are numerous reports of caveolin scaffolding domain interactions (Bernatchez et al., 2011; Bucci et al., 2000; Feron et al., 1998; García-Cardeña et al., 1997; Hatakeyama et al., 2006; Levin et al., 2007; Zhu et al., 2004), we find that the motif forms a key β strand element within the hydrophobic interior of the protein. The aromatic side chains are tightly packed in the protein core and, even more strikingly, directly contact the critical heme group within the protein's active site (Figure 2). It is extremely unlikely that this sequence could bind to caveolin without dramatic and detrimental conformational changes occurring. Similar observations can be made for the majority of other proteins for which structural data is available.

Could Conformational Changes Facilitate Caveolin Binding?

One possibility we considered was that the CBMs could become accessible upon conformational changes in the target proteins. This also appears unlikely in view of the critical structural roles of the majority of these peptides. Invoking the hypothesis that a conformational change could lead to binding may be a reasonable explanation, perhaps, for a single or small number of binding events (although notably there is currently no data to support such a model). However, given the large range of different proteins from diverse structural and functional classes we have examined here, conformational change in the signaling molecules appears highly implausible as a universal explanation.

Table 1. Putative Caveolin Binding Motifs (CBMs) Identified in Previous Studies[a]

Protein	Proposed CBM(s)	Reference[b]	Protein for Structural Analysis	PDB ID	Structure Reference	Secondary Structure	Accessible Surface Area (%)[c]
Consensus CBM	**Ω**x**Ω**xxxx**Ω**xx**Ω**	(Couet et al., 1997)	–	–	–	–	–
Soluble Proteins							
β-catenin	**Y**T**Y**EKLL**W**	(Mo et al., 2010)*	β-catenin	2Z6G	(Xing et al., 2008)	α helix	36
ApoE	**W**ELALGR**FW**D**Y**LR**W**[31]	(Yue and Mazzone, 2011)	ApoE	1YA9	(Hatters et al., 2005)	α helix	32
BTK	**W**A**F**GVLM**W**EI**Y**[591]	(Vargas et al., 2002)	BTK	1K2P	(Mao et al., 2001)	α helix	1
Dystrophin	**F**H**Y**DIKI**F**NQ**W**	(Couet et al., 1997)	none	–	–	–	–
eNOS	**F**PAAP**F**SG**W**[356]	(Couet et al., 1997; García-Cardeña et al., 1997; Sato et al., 2004)*	eNOS	1M9K	(Rosenfeld et al., 2002)	β strand	11
Gα subunits	**F**T**F**KDLH**F**KM**F**[199]	(Couet et al., 1997; Li et al., 1995)	$G\alpha_i1$	1CIP	(Coleman and Sprang, 1999)	β hairpin	32
			$G\alpha_q$	3AH8	(Nishimura et al., 2010)	β hairpin	44
GFP[d]	**F**A**Y**GVQC**F**SR**Y**	this study	GFP	3OGO	(Kubala et al., 2010)	extended coil	–
Heme oxygenase-1	**F**LLNIEL**F**[214]	(Taira et al., 2011)	heme oxygenase-1	1DVE	(Sugishima et al., 2000)	α helix	18
LC3B	**F**L**Y**MV**Y**ASQET**F**[119]	(Chen et al., 2010)	LC3B	2ZJD	(Ichimura et al., 2008)	β strand	13
MAP kinase	**Y**IVGF**Y**GA**F**[133]	(Couet et al., 1997)	MEK1	3PP1	(Dong et al., 2011)	β strand	21
Myosin HC	**W**P**W**MKLY**F**[836]	(Couet et al., 1997)	myosin HC	2MYS	(Rayment et al., 1993)	α helix	51
NSF	**F**S**F**NEKL**F**[145]	(Couet et al., 1997)	NSF	1QCS	(Yu et al., 1999)	β hairpin	38
Nuclear erythroid 2 p45-related factor2	**F**GDEF**Y**SA**F**[289]	(Li et al., 2012)	–	–	–	–	–
PKCα	**F**S**Y**VNPQ**F**[663]	(Oka et al., 1997)	PKCα	3IW4	(Wagner et al., 2009)	β strand	32
PPARγ[e]	**F**GD**F**MEPK**F**E**F**[370]	(Burgermeister et al., 2011)*	PPARγ	3ETO	(Artis et al., 2009)	α helix	23
PTEN	**F**H**F**WVNT**F**[278]	(Caselli et al., 2002; Xia et al., 2010)*	PTEN	1D5R	(Lee et al., 1999)	β strand	7
PTP1B	**F**HYTT**W**PD**F**[182]	(Caselli et al., 2002)	PTP1B	2CM2	(Ala et al., 2006)	β strand	28
PTP1C (SHP-1)	**F**V**Y**LRQP**Y**[213]	(Caselli et al., 2002)	SHP-1	3PS5	(Wang et al., 2011)	β strand	32
SH-PTP2	**W**Q**Y**HFRY**W**[423]	(Caselli et al., 2002)	SH-PTP2	3B7O	(Barr et al., 2009)	β strand	15
Src family kinases	**W**S**F**GILL**Y**[430]	(Couet et al., 1997)	Abl	2G2I	(Levinson et al., 2006)	α helix	3
Thioredoxin reductase 1	**Y**HS**YFW**PLE**W**[411]	(Volonte and Galbiati, 2009)*	thioredoxin reductase 1	3QFA	(Fritz-Wolf et al., 2011)	β strand	15
Transmembrane Proteins							
ALK1	**W**A**F**GLVL**W**[406]	(Santibanez et al., 2008)	ALK1	3MY0	–	α helix	1.8
Adiponectin receptor R1	**F**VPWL**Y**YS**F** (1), **F**FPGK**F**DI**W** (2)	(Wang et al., 2012)*	none	–	–	–	–
Angiotensin	**Y**G**F**LGKK**F**KR**Y**	(Wyse et al., 2003)	β1AR	2YO1	(Warne et al., 2011)	α helix	–
Aquaporin	**W**I**F**WVGP**F**[219]	(Couet et al., 1997)	AQP1	1J4N	(Sui et al., 2001)	α helix	22[f]
Caveolin	**F**TVTK**Y**WF**Y**	(Couet et al., 1997)	none	–	–	–	–
D1 dopamine	**F**DVFV**W**FG**W**	(Kong et al., 2007)*	none	–	–	–	–
Desmogleins	**F**CQKA**Y**A**Y**	(Brennan et al., 2011)	none	–	–	–	–
Endothelin R	**W**P**F**DHND**F**GV**F**	(Couet et al., 1997)	none	–	–	–	–
Receptor tyrosine kinases	**W**S**Y**GVTV**W**[881]	(Couet et al., 1997;	EGFR	3LZB	(Fidanze et al., 2010)	α helix	2
		Nystrom et al., 1999;	ephrin A3	2QOB	(Davis et al., 2008)	α helix	1
		(Vihanto et al., 2006)*	insulin R	1IRK	(Hubbard et al., 1994)	–	–
IP_3R3	**W**KINL**F**MQ**F**[226]	(Sundivakkam et al., 2009)	IP_3R1	1XZZ	(Bosanac et al., 2005)	β strand	32
mAcR	**W**TIGY**W**LC**Y**	(Couet et al., 1997)	none	–	–	–	–

(*Continued on next page*)

Table 1. *Continued*

Protein	Proposed CBM(s)	Reference[b]	Protein for Structural Analysis	PDB ID	Structure Reference	Secondary Structure	Accessible Surface Area (%)[c]
Maxi-K channel α subunit	YNMLCFGIY[1007]	(Alioua et al., 2008; Brainard et al., 2009)*	Maxi-K cytoplasmic domain	3MT5	(Yuan et al., 2010)	β strand	10
mGluR1α	FVTLIFVLY (1), FNEAKYIAF (2)	(Hong et al., 2009)*	none	–	–	–	–
MuSK	WAYGVVLWEIF[795], FSYGLPQY	(Hezel et al., 2010)	MuSK	1LUF	(Till et al., 2002)	α helix	3
Na/K ATPase[g]	FCRQLFGGF[93] (1), WWFCAFPY[987] (2)	(Cai et al., 2008)	Na/K ATPase	3B8E	(Morth et al., 2007)	α helix/ extended coil	37[f] (1), 25[f] (2)
Neu3 sialidase	YTYYIPSW	(Wang et al., 2002)	none	–	–	–	–
nAChR α subunit	FSFLTGLVFY[234]	(Hezel et al., 2010)	nAchR	2BG9	(Unwin, 2005)	α helix	19[f]
P-glycoprotein	FSMFRYSNW[44]	(Jodoin et al., 2003)*	P-glycoprotein	3G61	(Aller et al., 2009)	α helix/ extended coil	42[f]
Patched	YDFIAAQFKYF	(Karpen et al., 2001)*	none	–	–	–	–
TLR4	FIQSRWCIF[715]	(Wang et al., 2009)	TLR2	1FYW	(Xu et al., 2000)	α helix/ extended coil	29
TRPC1	FRTSKYAMF	(Sundivakkam et al., 2009)	none	–	–	–	–
β1 adrenergic receptor	FVFFNWLGY[333]	(Couet et al., 1997)	β1AR	2YO1	(Warne et al., 2011)	α helix	31[f]
Viral and Other Pathogen Proteins							
Cholera toxin subunit A	YGWYRVHF[132]	(Couet et al., 1997)	cholera toxin subunit A	1S5E	(O'Neal et al., 2004)	β strand	28
gp41	WNNMTWMQW[115]	(Hovanessian et al., 2004; Huang et al., 2007)	gp41	1QBZ	(Yang et al., 1999)	α helix	43
M2 channel	FFKCIYRRF[54]	(Zou et al., 2009)	M2 channel	2RLF	(Schnell and Chou, 2008)	α helix/ extended coil	64[f]
Matrix (M) protein	FGKSNWGLF	(Ravid et al., 2010)	none	–	–	–	–
Matrix protein	FCSAEWPTF[45]	(Yu et al., 2006)	murine leukemia virus matrix protein	1MN8	(Riffel et al., 2002)	α helix	29
α-hemolysin	WGPYDRDSW[187]	(Pany et al., 2004)	α-hemolysin	3ANZ	(Tanaka et al., 2011)	extended coil	41[f]

[a]The CBMs are derived from published studies that show association between caveolin and the identified proteins. These studies suggest that direct caveolin binding occurs via the indicated CBM, related to the sequence originally proposed by Couet et al., 1997. There were two motifs identified, ΩxΩxxxxΩ and ΩxxxxΩxxΩ, where Ω is Tyr, Phe, or Trp. Couet et al. further proposed a combined CBM of ΩxΩxxxxΩxxΩ.
[b]Asterisks (*) indicate that mutagenesis of the CBM was carried out.
[c]The accessible surface area is expressed as an average percentage of the total possible surface area of each side chain within the CBM sequence. Areas were calculated using the program NACCESS (http://www.bioinf.manchester.ac.uk/naccess/).
[d]Note that the GFP sequence encompasses the cyclized Tyr that forms the chromophore.
[e]Note that the sequence in PPARα (and also PPARα,β,δ) is actually in the reverse sequence orientation to the consensus CBM.
[f]For these indicated transmembrane proteins, "accessible surface area of CBMs" will not be an accurate indication of solvent exposure, as the sequences lie within membrane spanning regions or extracellular domains (Figure S2).
[g]Note that both sequences fall within the transmembrane domain.

Could the proteins interact with caveolin after synthesis but before adopting a fully folded structure? We cannot rule out this possibility, but it would almost certainly give rise to a nonfunctional stable association with caveolin that would not be subject to the dynamic regulation required during cell signaling.

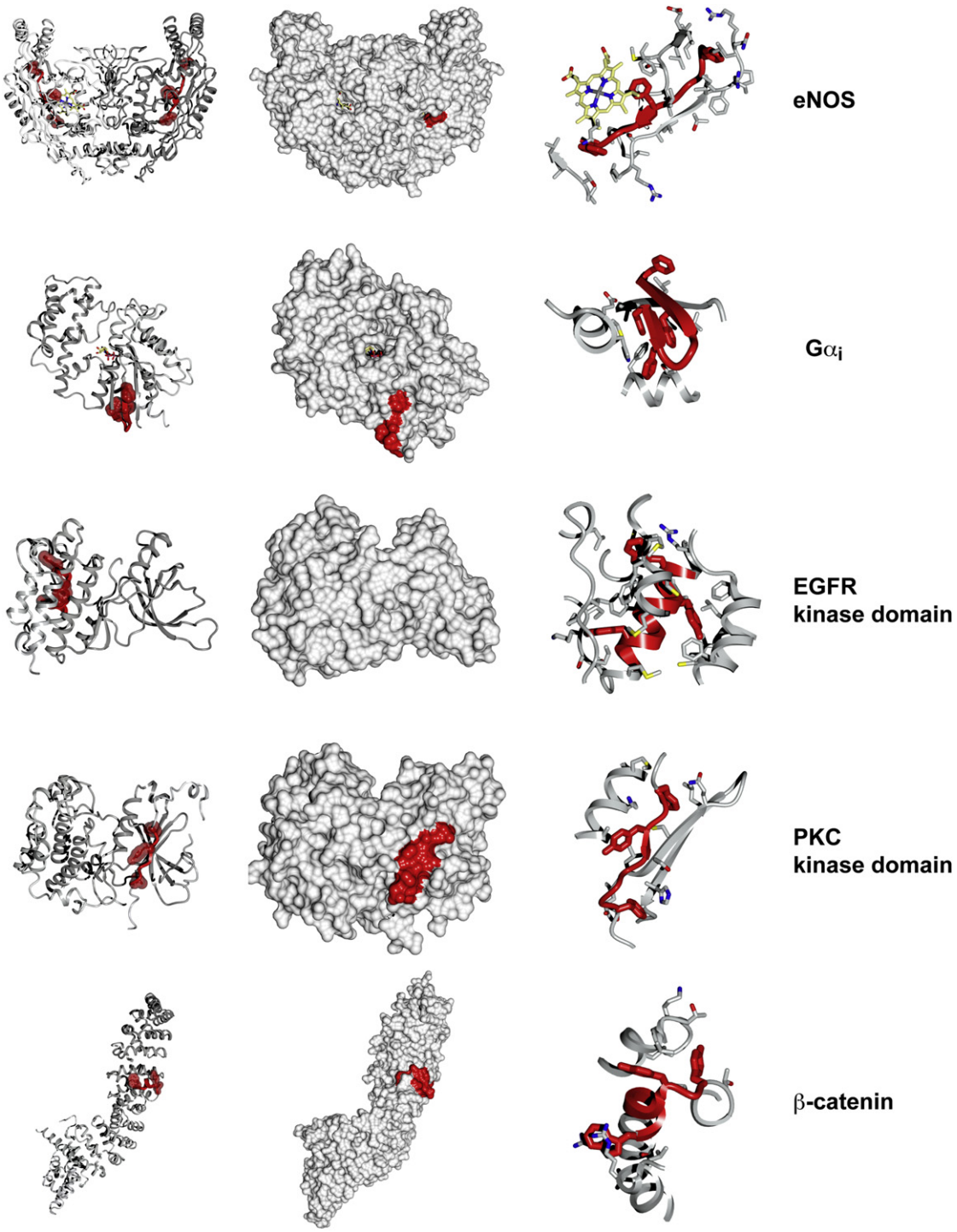

Figure 2. Structural Comparison of Several Examples of Putative Caveolin-Interacting Proteins

An enhanced animation of the eNOS structure is provided in Movie S1. Left panels show proteins in ribbon representation, with the CBM indicated in red. Key aromatic residues of the putative CBMs are highlighted in surface representation. In each case, the key aromatic residues are tightly packed within the protein hydrophobic core. Middle panels show the same views in surface representation, with CBMs indicated in red. The right panel shows a close-up view of the CBM and the surrounding environment. Key aromatic residues of the CBMs are shown in red stick representation, and side chains forming direct intramolecular contacts with these aromatic CBM residues are indicated in gray stick representation. For eNOS, the core heme group is shown in yellow stick representation. All known structures of putative caveolin-interacting proteins are shown in Figure S2, with references in Table 1. All structure images were prepared using CCP4mg (McNicholas et al., 2011).

Table 2. Bioinformatic Analysis of the Abundance of Consensus CBMs in Mouse and Yeast Proteins[a]

	Number of Proteins	Number Containing CBM	Overall Percentage (%)
Mouse			
Full-length proteins[b]	33,451	10,076	30
Cytoplasmic sequences[c]			
Soluble	22,265	5,936	27
Type I transmembrane	1,548	201	13
Type II transmembrane	2,869	335	12
Multipass transmembrane	3,821	739	19
Total	30,503	7,211	24
Noncytoplasmic sequences[c]			
Soluble	2,948	996	34
Type I transmembrane	1,548	488	32
Type II transmembrane	2,869	608	21
Multipass transmembrane	3,821	773	20
Total	11,186	2,865	26
Yeast			
All proteins	6,736	2,883	43

[a]Sequences derived from CYGD database (http://mips.helmholtz-muenchen.de/genre/proj/yeast/) were scanned for the presence of any of the two putative CBM sequences, ΩxΩxxxxΩ and ΩxxxxΩxxΩ, or the combined consensus sequence ΩxΩxxxxΩxxΩ (Couet et al., 1997), where Ω is either Phe, Trp, or Tyr.4

[b]The full set of 51,135 coding sequences was reviewed, and those with annotated truncations at the N terminus were discarded; topology with respect to the membrane cannot be accurately determined in this set.

[c]Topology with respect to the membrane was calculated based on the presence in sequences of signal peptides and integral membrane domains using a previously published annotation pipeline (Davis et al., 2006).

Caveolin Binding Motifs Are Not Enriched in Caveolae-Associated Proteins

This analysis raises the question of why so many proteins, particularly signaling proteins, which have been proposed to interact with caveolins, possess CBMs. In fact, a systematic bioinformatics analysis of full-length coding sequences from the entire mouse genome (Carninci et al., 2005) reveals that this motif is actually present in 30% of all proteins, irrespective of localization or function (Table 2). The motif is not enriched (and is in fact less abundant) in cytoplasmic proteins and the cytoplasmic regions of transmembrane proteins that might conceivably bind caveolin at the inner leaflet of the plasma membrane. Perhaps most tellingly, the motifs show even greater prevalence in the genome of *Saccharomyces cerevisae*, which lacks caveolins altogether. Thus, it is clear that CBM sequences are not enriched in caveolae-associated molecules, and their widespread abundance likely reflects a common requirement for hydrophobic aromatic side chains in protein hydrophobic cores or transmembrane segments for structural stability and function.

In summary, it is clear from the available structural and genomic data that the proposed ΩxΩxxxxΩxxΩ CBM sequences are unlikely to represent a conserved peptide motif for direct recognition of the caveolin scaffolding domain. Another factor to consider when assessing the viability of the proposed caveolin interaction is the position of the putative CBM in the protein, with respect to the membrane in which caveolin is embedded. An analogous example is the recognition of tyrosine-containing motifs by clathrin adaptors, which must be further than 7 amino acids from the membrane interface to engage with cytoplasmic proteins (Rohrer et al., 1996). This immediately raises an additional point regarding interactions with caveolin, because the maximum distance of the central-most portion of the CSD from the membrane—assuming a completely and unrealistically extended structure—is only 30 Å, corresponding to 10 amino acids (Figure 1). This will impose severe steric constraints on any interactions with putative binding partners, which have been reported to be cytoplasmic proteins, cytoplasmic domains of transmembrane proteins, or even extracellular membrane penetrating polypeptides (e.g., gp41, Hovanessian et al., 2004).

Implications for the Caveolin Signaling Model

Mutations in caveolins or caveolin deficiency can clearly influence many signaling pathways, as shown both in vitro and in vivo, and there is no doubting the role of caveolins in numerous cellular functions. The signaling proteins listed in Table 1, as well as many other molecules, can be immunopurified in caveolin-enriched membrane fractions. However, experiments in which signaling proteins associate with caveolin, as judged by immunoprecipitation, must be viewed with caution, given the poor solubility of caveolin-enriched domains (as discussed by Parton and Simons, 2007), and do not necessarily indicate a direct protein-protein interaction. A number of studies have assessed the effect of either deleting or mutating the CBM on caveolin association and in signaling assays (see Table 1) and have generally shown a disruption in caveolin interaction and function. However, the loss of an apparent interaction through mutation of the proposed CBM will be highly misleading if protein folding, trafficking, or microdomain localization is disrupted, as seems highly likely given the critical structural roles of the majority of CBM sequences. Very few reports have addressed the localization or expression of mutant signaling proteins. The mutant zebrafish β-catenin protein was found to at least localize to the nucleus similarly to the wild-type (WT) molecule (Mo et al., 2010), and the mutant Maxi-K potassium channel α subunit (Slo1) showed similar sedimentation and oligomeric properties to the WT protein in sucrose gradients (Alioua et al., 2008). In contrast, the mutant EphB1 receptor tyrosine kinase was expressed at lower levels than the WT protein and was not localized to the plasma membrane (Vihanto et al., 2006). Structural integrity and correct protein folding has not been tested for any of the mutant proteins, to the best of our knowledge, and should certainly be a priority in future studies.

The inhibition of signaling processes by cell-permeable peptides corresponding to the caveolin scaffolding domain (amino acids 82–101 in caveolin-1) represent an additional line of evidence supporting the original caveolin scaffolding hypothesis. These studies have demonstrated a striking effect of this peptide on key signaling pathways involving proteins such as eNOS, phospholipase D (PLD), and Rac1, both in cultured cells and in tissues (Bernatchez et al., 2005; Czarny et al., 1999; Gratton et al., 2003; Kim et al., 1999; Nethe et al., 2010). In animal

models, administration of the caveolin-derived peptide reduced the permeability of the tumor vasculature and delayed tumor progression, an effect that was reduced in mice lacking the putative target, eNOS (Gratton et al., 2003). Conversely, a noninhibitory version of this peptide with a single amino acid change increases basal NO release, an effect lost in tissues lacking eNOS or caveolin-1 (Bernatchez et al., 2011). However, only a limited number of studies have attempted to directly test binding of the CSD peptide to signaling proteins, and in these cases binding was not investigated in the context of an interaction with putative CBMs (Kim et al., 1999; Nethe et al., 2010). The analyses presented here should prompt reinvestigation of the mechanisms involved in inhibition of signaling by these peptides and, more generally, the effect of loss of caveolin and/or caveolae on specific signaling pathways. Our findings certainly do not preclude the regulation of signaling pathways by caveolins through other mechanisms. These may include interactions mediated by other regions of caveolin (such as the interaction with phosphorylated caveolin-1 on tyrosine 14; Chen et al., 2012; Place et al., 2011) or by completely independent mechanisms, including effects on lipid-based organization of the plasma membrane (Gaus et al., 2006; Hoffmann et al., 2010) or endocytosis (Cheng et al., 2010; Kirkham et al., 2005). These effects are also abrogated by mutations in the caveolin scaffolding domain (Cheng et al., 2010; Hoffmann et al., 2010).

Taken together, the findings presented here argue against a role for caveolin binding motifs in driving direct protein recruitment to caveolae. The putative CBM sequence is not enriched in proteins associated with caveolae, and the motif does not adopt a common binding structure and is not exposed for caveolin binding. In most cases, the CBM is part of a critical structural element, the perturbation of which is likely to lead to protein misfolding. We suggest that these considerations must be taken into account in future studies of caveolin interactions. In addition, previous work implicating caveolin as a scaffold for direct protein recruitment may need to be reassessed to reveal the actual mechanisms by which caveolins modulate specific signaling pathways.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.06.012.

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